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Determination of Diarrhetic Shellfish Poison by Measuring 50% Acid Phosphatase Activity Inhibitory Concentration ($APIC_{50}$)

(Determination of Diarrhetic Shellfish Poison by Using *Tetrahymena pyriformis*, 2)

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A practical determination of diarrhetic shellfish poison (DSP) was established by measuring the 50% acid phosphatase activity inhibitory concentration ($APIC_{50}$) with *Tetrahymena pyriformis*, in place of the mouse lethal assay. That is, DSP was extracted with acetone and ether from the toxic hepatopancreas of scallops, and applied to a silicic acid column. The column was washed with ether-methanol (40:1), and DSP was eluted with ether-methanol (1:1). A suitable concentration of DSP was added to 20 ml of the medium for growth of *T. pyriformis* in an Erlenmeyer flask. This flask was inoculated with 0.2 ml (approximately 4,000 cells) of preculture of *T. pyriformis* and incubated at 28°C for 24 hours. The cultured cells were washed twice with 0.5% saline, and destroyed by sonication for 5 min. Then the acid phosphatase activity in *T. pyriformis* was determined.

The $APIC_{50}$ and the 50% growth inhibitory concentration (GIC_{50}) of *T. pyriformis* were equivalent to 1 Mouse Unit (0.05 MU/g of sample). The presence of DSP in samples can be judged qualitatively by using the naked eye when the toxin content is above the GIC_{50} of *T. pyriformis*. For the analysis of a few samples, it is convenient to apply GIC_{50} for quantitation of DSP, but the application of $APIC_{50}$ is preferable for the treatment of many samples.

(Received June 21, 1985)

Key words: 下痢性貝毒 diarrhetic shellfish poison; 50% 酸性ホスファターゼ活性阻害値 50% acid phosphatase activity inhibitory concentration; テトラヒメナ *Tetrahymena pyriformis*; ホタテ貝 scallop; 50% 増殖阻害値 50% growth inhibitory concentration

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Foreword

We reported through basic tests[1] that the growth of tetrahymenae is specifically inhibited by diarrhetic shellfish poison, that the 50% growth inhibitory concentration (GIC_{50}) corresponds roughly to 0.5 mouse unit/10 mL of medium (abbreviated hereafter as MU), and that it is therefore possible to avoid all kinds of defects inherent to the mouse method by using tetrahymenae to determine diarrhetic shellfish poison.

However, 3 days were needed to know the GIC_{50} of the tetrahymenae because we used the test tube still culture method. Moreover, because we used a hematocyte counting cell to count the cells, the operation was cumbersome. So, using this method as it was in routine examinations was difficult. /p. 639

This time, we tried to shorten the tetrahymena culture time and to find simplifications based on the measurement of acid phosphatase activity produced by the tetrahymenae. We obtained results which may be used in practical daily examinations. We report hereunder about these results.

Material and testing method

1. Samples

We used the same poisoned scallop midgut glands as in our former report[1].

2. Reagents

Acetone, ether, methanol, formalin: same as in our former report[1)]

Column chromatography: as in our former report[1)]

Medium: same as in our former report[1)]

Acid phosphatase reagent: we used a Wako acid phosphatase B kit

3. Equipment

Ultrasoundwave crusher: Tomi UR 2000 P Sonicator

Centrifuge: Tomi Seiko Co. CD-50 SW

Spectrophotometer: Hitachi Seisakusho Model 101

Other equipment: same as in former report[1)]

4. Purification of diarrhetic shellfish poison

After extracting the sample by the official method[2)] we used the partially purified diarrhetic shellfish poison of the portion obtained by ethanol-methanol (1:1) elution from a silicic acid column similarly to our former report[1)] and employed it for the following tests.

5. Measuring the poison quantity

We performed mouse lethal tests as in our former report[1)].

6. Tetrahymenae

Same as in the former report[1].

7. Method of culturing the tetrahymenae

Tetrahymenae require oxygen in order to grow. So, to increase the contact surface between the air and medium, we changed the "test tube: 10 mL medium" used in the former report[1] to "100 mL conical flask: 30 mL medium". Aside from this, the method was exactly the same as in our former report[1].

8. Tetrahymenae growth inhibition test based on the shellfish poison fraction

i. Method based on growth cell count

We put in sterilized test tubes shellfish poison fractions of known poison volume measured through mouse lethal tests. After allowing the solvent to evaporate in a 80° dry block bath, we added the medium aseptically and prepared a 1 MU/mL suspension. Adding 0, 0.5, 1, 1.5, 2 mL of the above suspension to the 100 mL conical flask, we prepared a 0, 0.025, 0.05, 0.075, 0.1 MU/mL toxin series. We inoculated 0.2 mL of each of the above tetrahymena cultures (about 4,000 cells), and performed still culture at 28°C for 24 h. We measured the growth inhibition rate through cellular count as in our former report[1].

ii. Method based on acid phosphatase activity measurement

We added 4 drops of 3.7% formalin to the medium solution obtained as in 8.i, centrifuged for 5 min at 2000 rpm. For the extracellular acid phosphatase sample solution, we washed twice the supernatant with a 0.5% common salt solution, and for the intracellular acid phosphatase sample solution, we washed the precipitated tetrahymenae in the same manner. Thereafter, we sonicated at the highest output for 5 min, and used the supernatant separated through centrifugation at 2000 rpm for 5 min. For the total acid phosphatase, sample solution, we sonicated the medium solution for 5 min at the maximum output, centrifuged for 5 min at 2000 rpm and used the supernatant.

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iii. Method of washing the tetrahymenae

In order to measure the intracellular acid phosphatase, one must first remove the extracellular phosphatase. We used 1.05, 0% common salt solutions as wash for the cells and examined the numbers of washings based on the centrifugal method with regard to the various concentrations.

iv. Method of measuring the acid phosphatase activity

We followed the indications given by the kit we purchased. That is, we added 0.5 mL of substrate buffer to 0.1 mL of test solution and reacted for 30 min at 37°C. Thereafter, we added 5 mL of 0.05 N sodium hydroxide solution, allowed development and measured at a 405 nm

wavelength. For the acid phosphatase activity we used the Bessey-Lowry units (BL unit). One BL unit is the volume of enzyme producing 1 nM of p nitrophenol through a 37°C and 1 h action on the substrate. The substrate buffer had been obtained by dissolving 12.3 mg of p nitrophenyl phosphate disodium per 5 mL of 0.05 M citric acid buffer (pH 4.8).

Results and discussion

1. Method of growing the tetrahymenae

Fig. 1 shows the growth curve of the tetrahymenae an the extracellular acid phosphatase activity.

When using '100 mL conical flask: 20 mL medium' the cellular growth shows an exponential growth up to 17-72 hours. The number of cells in 24 h of culture reached the 10^4 order just as with 48 h, 72 h of 'test tube: 10 mL medium' culture (we extrapolated the Fig. of the former report. These results show that the culturing time may be shortened to 24 h. Placing the acid phosphatase activity on the right ordinate of Fig. 1 in BL units, we plotted the extracellular acid phosphatase activity isolated in the culture solution. The curve obtained was practically parallel to the growth curve of the tetrahymenae. These results suggest that the cumbersome cell number counts may be replaced by the simple acid phosphatase measurement method.

2. Tetrahymena growth inhibition caused by diarrhetic shellfish poison

Fig. 2 shows the results obtained by examining the growth inhibition of tetrahymenae in the shellfish poison fraction.

The abscissa denotes the poison volume (MU) per 20 mL of medium and the lower level figures denote the MU converted values per 1 g of sample. 0.05 MU/g is the regular standard of poisoning. The ordinate is the relative growth rate in poison-containing medium when the cell number of tetrahymenae grown in a culture containing no poison is set at 100. Like in the 72 h 'test tube: 10 mL medium' culturing method[1], the tetrahymena GIC_{50} was in practical agreement with the regular poisoning standard value of 0.05 M/g in the '100 mL conical flask: 20 mL medium' culturing method. /p. 640

3. Diarrhetic shellfish poison determination based on acid phosphatase activity

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In the determination method based on measuring the cell counts of tetrahymenae, one utilizes a microscope and several cell counts are made per one specimen. This is why the method is cumbersome and unpractical when there are many samples.

We noticed that the acid phosphatase produced by the tetrahymenae is a stable enzyme and that a highly sensitive measurement method had been secured, and we examined a determination method for diarrhetic shellfish

poison based on the measurement of tetrahymena acid phosphatase activity as a method requiring no labour. We also compared the intracellular, extracellular and total acid phosphatase activity inhibitions.

i. Washing the tetrahymenae

To measure the intracellular acid phosphatase activity, we washed the cells and flushed the intracellular acid phosphatase by sonication. There was however no concern about disorder in the measurement values caused by the destruction of cells in the washing process. We examined the washing conditions of the tetrahymenae. The results are shown in Fig. 3.

By assuming 100% for intracellular acid phosphatase activity on the ordinate, the washing efficiency was 99.1% when washing twice with 30 mL of 1% and 0.5% common salt solutions, and 95.2% when washing with distilled water. The reason why the washing efficiency is slightly poor with distilled water seems to be that part of cells had broken.

We choose therefore to wash twice with 30 mL of 0.5% common salt solution.

The acid phosphatase activity of the tetrahymenae /p. 641

Fig. 4 shows the results obtained by comparing the extracellular, intracellular and total acid phosphatase activities and the MU.

The abscissa represents the volume of poison, and the ordinate represents the relative activity rate when putting the acid phosphatase activity of the control (contains no poison) at 100%. When we have a 1 MU acid phosphatase activity, the extracellular acid phosphatase activity is 69.9% (n=7, CV=0.8%), the total acid phosphatase activity is 64.2% (n=7, CV=4.2%) and the intracellular acid phosphatase activity is 51.8% (n=7, CV=4.3%). Of the three, it is the intracellular acid phosphatase which agreed best with 1 MU/20 mL of medium (regular value of 0.05 MU/g).

It seems that the results obtained may be substituted to the expression saying that the 50% acid phosphatase activity inhibition concentration ($APIC_{50}$) corresponds to the normal 0.05 MU/g value. As the mouse lethal method determines the volume of diarrhetic shellfish poison by taking the end point as standard, it is difficult to know the exact quantity of poison. More precise determinations are obtained by taking as standard $APIC_{50}$ which agrees well with the regular value of 0.05 MU based on the mouse lethal method.

We summarize the above results by saying that we established a practical diarrhetic shellfish poison inspection method based on tetrahymenae, a method which as routine examination method is more precise, simpler and less expensive. We present it in Scheme 1.

We used the present method for 20 commercial specimens of short-necked clams, scallops and made the same detections as in the mouse lethal method.

Conclusion

We established a method based on the $APIC_{50}$ measurements of tetrahymenae to replace the mouse lethal test method which is the sole inspection method for diarrhetic shellfish poison. That is, we extracted successively by acetone, ether the poison from the midgut glands of poisoned scallops, used silicic acid columns, washed with ether-ethanol (40:1) and added the shellfish poison eluted with ether-methanol (1:1) to 100 mL conical flasks containing 20 mL of medium.

This was inoculated with 0.2 mL (cell number of about 4,000) of tetrahymena preculture solution, and we cultured at 28°C for 24 h. We washed the cultured cells with a 0.5% common salt solution, crushed by ultrasound waves and measured the acid phosphatase activity. We used the $APIC_{50}$ as diarrhetic shellfish poison determination value.

1. The GIC_{50} and $APIC_{50}$ agreed with 1 MU (regular value of 0.05 MU/g of specimen).

2. With respect to qualitative analysis, it is possible to observe macroscopically the culture solution if the amount of poison is above GIC_{50}.

3. When the number of samples is small, it is possible to determine precisely with GIC_{50}. When the number is large, the same is true with $APIC_{50}$.

The above demonstrates that the present method is as a routine

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method of diarrhetic shellfish poison examination simpler, cheaper and more precise than the mouse lethal method.

An outline of the present study was presented at the 48th scientific symposium of the Japanese Food Hygiene Society (October 1984, Fukui).

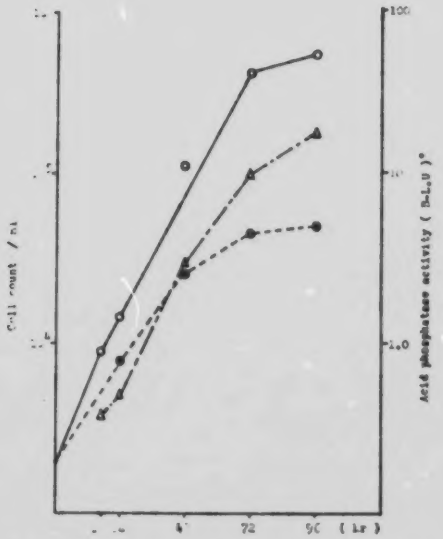

Fig. 1. Growth curve of *T. pyriformis* in the medium and acid phosphatase activity
○——○: cell counts by the proposed method, medium: 20 ml in Erlenmeyer flask (volume 100 ml); ●- - -●: cell counts by the previous method[1], medium: 10 ml in test tube (18×180 mm); △— · —△: extracellular acid phosphatase activity by the proposed method, medium: 20 ml in Erlenmeyer flask (volume 100 ml); incubation: standing condition at 28°C
* B.L.U: Bessey-Lowry Unit

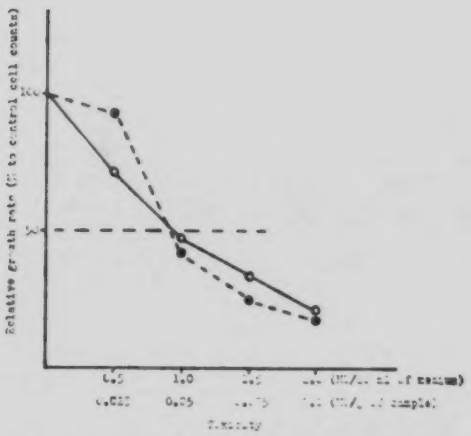

Fig. 2. Growth rate of *T. pyriformis* and potency of diarrhetic shellfish poison
○——○: proposed method, medium: 20 ml in Erlenmeyer flask (volume 100 ml); ●- - -●: previous method[1], medium: 10 ml in test tube (18×180 mm); incubation: standing condition at 28°C.

/p. 642

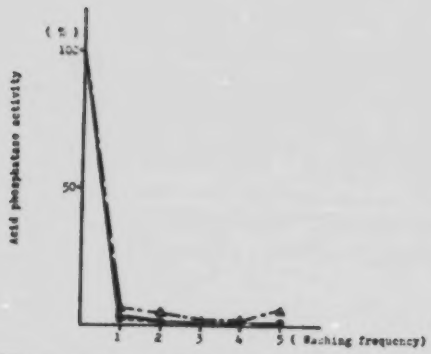

Fig. 3. Removal effect of washings on extracellular acid phosphatase activity
○——○: 1% NaCl; ●- - -●: 0.5% NaCl;
△—·—△: distilled water
Each washing volume was 30 ml per frequency.

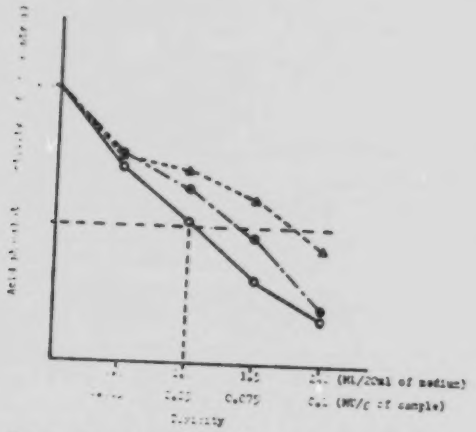

Fig. 4. Relationship between acid phosphatase activity and the potency of diarrhetic shellfish poison
○——○: intracellular; ●—·—●: whole;
△- - -△: extracellular

Medium (20 ml in an Erlenmeyer flask)
- inoculate at desired concentration ←
- inoculate 4,000 cells (0.2 ml of culture) of *T. pyriformis*
- incubate at 28°C for 24 hr

Culture
- for test of a few samples, apply GIC_{50} method, that is, add several drops of 3.7% formalin for cell counts of *T. pyriformis* on Bürker Turk slide glass under microscope *
- for test of many samples, wash the culture with 30 ml of 0.5% saline, twice, by centrifugation at 2,000 rpm for 5 min **

Suspension in 20 ml of 0.5% saline
- sonicate for 5 min
- centrifuge at 2,000 rpm for 5 min

Supernatant
- 0.1 ml of supernatant
- 0.5 ml of *p*-nitrophenilphosphate disodium salt in citrate buffer
- incubate at 37°C for 30 min
- 5 ml of 0.05 *N* NaOH
- determine the optical density at 405 nm

$APIC_{50}$ (comparison with a negative control)

Hepatopancreas (25 g)
- homogenize with 75 ml of $(CH_3)_2CO$, twice

Homogenate
- filter through filter paper

Acetone layer
- concentrate on a rotary evaporater at ambient temperature
- partition between ether and water

Ether layer
- wash with water, 3 times
- concentrate to dryness on a rotary evaporater at ambient temperature

Residue
- dissolve in a small volume of ether

Silicic acid column (alkali treatment)
- eluate with 250 ml of Et_2O-MeOH (40:1) ①
- eluate with 250 ml of Et_2O-MeOH (1:1) ②
- eluate with 250 ml of MeOH ③

Eluate ① Eluate ②
- concentrate to dryness, similarly

Residue
- suspend in 1 ml of growth medium for *T. pyriformis*

Suspension

Scheme 1. Determination of diarrhetic shellfish poison by using *T. pyriformis*
* Existence of DSP can be judged in comparison with a negative control.
** GIC_{50} can be determined in good precision in comparison with a negative control. (correspond to 1 MU of toxicity).

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